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Neuroprotective Effects of *Withania somnifera* on 4-Hydroxynonenal Induced Cell Death in Human Neuroblastoma SH-SY5Y Cells Through ROS Inhibition and Apoptotic Mitochondrial Pathway

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Abstract

The antioxidant, anti-inflammatory, and anticancer activities of *Withania somnifera* (WS) are known for a long time. This study was aimed to examine whether WS also diminishes 4-hydroxy-trans-2-nonenal (HNE)-induced neurotoxicity in human neuroblastoma (SH-SY5Y) cell line. The cytotoxic response of HNE (0.1–50 μM) and WS (6.25–200 μg/ml) was measured by MTT assay after exposing SH-SY5Y cells for 24 h. Then neuroprotective potential was assessed by exposing the cells to biologically safe concentrations of WS (12.5, 25, and 50 μg/ml) then HNE (50 μM). Results showed a concentration-dependent protective effect of WS at 12.5, 25, and 50 μg/ml against HNE (50 μM) induced cytotoxicity and cell inhibition. Pre-exposure to WS resulted in a strong inhibition of 24, 55 and 83% in malondialdehyde (MDA) level; 5, 27 and 60% in glutathione (GSH) level; 12, 36 and 68% in catalase activity; 11, 33 and 67% in LDH leakage; and 40, 80 and 120% in cellular LDH activity at 12.5, 25, and 50 μg/ml, respectively, induced by 50 μM HNE in SH-SY5Y cells. The HNE-mediated cellular changes (cell shrinkage, rounded bodies, and inhibition of outgrowth) and increased caspase-3 activity were also prevented by WS. The HNE-induced upregulation of proapoptotic markers (p53, caspase-3, and -9, and Bax) and downregulation of antiapoptotic marker Bcl-2 genes were also blocked by pretreatment with WS. Altogether, our findings indicate that WS possesses a protective potential against HNE-induced neurotoxicity.

Keywords *Withania somnifera* · 4-hydroxy-trans-2-nonenal · SH-SY5Y · Cytotoxicity · Oxidative stress · Apoptosis

Introduction

Among the harmful by-products of lipid peroxidation (LP), 4-hydroxy-trans-2-nonenal (HNE) is a potentially dangerous product [1]. It remains stable in the lipid bilayer and subsequently diffuses through the membrane into the cytosol [2]. Studies have confirmed that aldehydes generated endogenously through the process of LP are informally involved in the etiology of numerous neurodegenerative ailments [3, 4]. HNE, a long-chain alpha, beta unsaturated aldehyde, is generated mainly by the oxidation of ω-6 polyunsaturated fatty acids [5]. Research showed that HNE causes cytotoxicity and oxidative stress-mediated cell death in many cell types, including SH-SY5Y cells [6–10]. It has been reported that HNE is highly reactive and stable [11, 12] and induces apoptosis in a variety of cells from different origins [13, 14]. We have also previously demonstrated that HNE induced cell death through oxidative damage and ROS generation in PC-12 cells [15–17]. Numerous other reports have also shown that HNE plays a precise role in signaling pathways, mitochondrial cytochrome c release, and caspase-9/3 activity [18, 19]. HNE is associated with progression of mitochondrial-dependent apoptosis and Bcl-2 family with various genes and their proteins [20]. Its neurotoxic property was verified based on the presence of high concentrations of HNE in nervous tissues and ventricular fluids of patients with Alzheimer's disease (AD), associated

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with augmented neuronal apoptosis [21, 22]. Elevated levels of HNE and it adducts have been found in the neurons of patients with Parkinson's disease (PD) [23], and also in Lewy bodies of these patients [24]. Numerous antioxidants are known to be capable of altering the intracellular level of HNE and preventing the progress of PD and AD [25]. Since, ancient time, several herbal remedies such as *Ginkgo biloba*, *Curcuma longa*, *Withania somnifera*, *Bacopa monnieri*, *Salvia officinalis* and *Panax ginseng* [26] used in conventional system have been reported to possess diverse activities in neuroprotection, memory enhancement, and antiaging. The extracts of the plant *Morus alba Linn* have been reported to possess neuroprotective potential against β-amyloid induced neurotoxicity [27]. Studies have also documented the neuroprotective potential of several other plants against toxic insult, such as *Ocimum sanctum* against H_2O_2-induced SH-SY5Y cell death [28], *Ginkgo biloba* (Ginkgoaceae) against MPTP-damaged nigrostriatal neurons in mice [29], *Cistanche deserticola* against 6-hydroxydopamine (6-OHDA)-instigated injury in rat neurotransmitters [30], and *Valeriana officinalis* against rotenone-induced apoptosis in human neuroblastoma SH-SY5Y cells [31]. Moreover, the neuroprotective effects of drugs derived from natural products e.g., resveratrol found in red grapes [16, 32], baicalein found in *Scutellaria baicalensis* (Labiatae), and curcuminoids found in *C. longa*, have been screened under both in vitro and in vivo conditions [33, 34]. These reports showed that natural extracts derived from herbal plants possess biologically active components of pharmacological importance and exhibit neuroprotective potential against various toxicants. *W. somnifera* (WS), a potent medicinal plant popularly recognized as Ashwagandha, has been used since ancient times in the traditional health care system [35]. Studies have reported the antioxidant [36], anticancer [37], anti-inflammatory [38], antistress [39], adaptogenic [40], antiarthritis [41] and immunomodulatory [42] activities of WS and its components. Whole or different parts of WS plant extracts have also been used for their beneficial effects on the nervous system [43]. Clinical and preclinical studies have supported the therapeutic potential of WS against cognitive and memory-related disorders [44]. Clinically WS has been confirmed to be an antidepressant and anti-anxiety agent [45]. It has been reported to be effective in patients diagnosed with PD [46]. The anxiolytic efficacy of ethanolic extract of WS has also been reported [47]. Our literature survey disclosed that toxicological studies of clinical research on WS have demonstrated that the plant is nontoxic at an extensive variety of applied doses [48–50]. Another study showed that WS reduced serum cortisol levels without causing any major side effects in humans [51]. However, the protective potential of WS against HNE-induced cell death has not yet been explored. Therefore, the present investigation was conducted to evaluate the neuroprotective effect of WS against HNE-induced neurotoxicity in SH-SY5Y cells, a human neuroblastoma cell line. This cell line has been demonstrated to be a very useful model system for studying neuronal differentiation and function and has been developed into a neuronal-like phenotype upon differentiation [52]. Similarly, the SH-SY5Y cell line is commonly used and represents a suitable experimental model system for investigating the molecular and cellular mechanisms-involved in studies on neurotoxicology and neuropharmacology [53]. In neuroscience research, the SH-SY5Y cell line has also been extensively used as an in vitro model system [54].

Material and Methods

Chemicals

All chemicals, reagents, solvents, toxicants, and cell culture medium of analytical grade were procured from Sigma, USA. Fetal bovine serum, antibiotic/antimycotic and trypsin were purchased from Gibco-Invitrogen.

Extraction of Plant

The medicinal plant, WS was collected from the local market, Lucknow, India. The aerial part was rinsed with distilled H_2O and air-dried under shade. The extraction was done by maceration. The dried powder was soaked in methanol, and the solution was filtered. The solvent was then concentrated in a rotary evaporator, and obtained WS extract was stored at 4 ºC until further dilution and bioassays.

Cell Culture

The human neuroblastoma cell line (SH-SY5Y) was grown in DMEM-F12 with 10% FBS, 0.2% sodium bicarbonate and 1% antibiotic/antimycotic solution. Confluent cells were trypsinized and cultured in plates and flasks as required for the experiments. Cells were cultured in a CO_2 incubator (37 ºC, 5% CO_2, 95% related humidity).

Cell Exposure to HNE and WS

SH-SY5Y cells were exposed to HNE (0.1, 1, 5, 10, 25, and 50 μM) and WS (6.25, 12.5, 25, 50, 100, and 200 μg/ml). The cytotoxicity was measured after 24 h exposure. For assessing cytoprotection, the cells were pre-exposed to medium containing WS (12.5, 25 and 50 μg/ml) for 24 h and then to HNE (50 μM) for 24 h.

MTT Assay

The MTT assay was done following the technique [17] to assess HNE- and WS-induced cytotoxicity. Approximately 10^4 SH-SY5Y cells were plated in a flat bottom 96-well culture plate and kept in the incubator. On the next day, the cells were treated with HNE and/or WS for 24 h, after which 10 µl of MTT was added to the wells and incubated for an additional 4 h. Formazan crystals formed were dissolved in 200 µl DMSO, and the plate was read at 530 nm. The experiments were performed in triplicate.

Neutral Red Uptake (NRU) Assay

This assay was achieved using the technique of Siddiqui et al. [17]. After the exposure, SH-SY5Y cells were incubated with 50 µg/ml of NR dye for 3 h in an incubator. Then, the dye was extracted and the wells were washed with a washing solution (1% $CaCl_2$ and 0.5% formaldehyde). Next, 200 µl of dye release solution (1% acetic acid and 50% ethanol) was added to each well and the plate was read at 550 nm. The experiments were performed in triplicate.

Trypan Blue (TB) Exclusion Assay

The TB exclusion assay was performed to assess the proportion of cell viability by estimating the membrane integrity of SH-SY5Y cells following the method of Pant et al. [55]. Briefly, after respective treatments, the cells were harvested, washed, and centrifuged. Then, the cells were stained with TB (0.4%) in a ratio of 1:5 of dye:cell suspension. Live and dead cells were counted using a hemocytometer. The experiments were performed in triplicate.

Lactate Dehydrogenase (LDH) Assay

LDH assay was performed using the LDH assay kit (Bio Vision) as per the instruction provided by manufacturer. This assay measures the level of lactate dehydrogenase released from damaged cells. The experiments were performed in triplicate.

Malondialdehyde (MDA) Level

MDA, the breakdown product of oxidation degradation of lipid cell membrane, is an indicator of lipid peroxidation. Following the procedure of Buege and Aust [56], the LP was estimated by determining the concentration of MDA in cells exposed to HNE and/or WS. The MDA concentration was calculated by measuring the developed color of TBARS at 532 nm. The experiments were performed in triplicate.

Glutathione (GSH) Content

The GSH content was estimated by determining the decrease of 5,5-dithiobis-2-nitrobenzoate (DTNB) to the yellow colored compound 2-nitro-5-thiobenzoate following the method [57]. The concentration of the yellow-colored formed was read at 412 nm that reflects the quantity of -SH groups. The experiments were performed in triplicate.

Catalase

Catalase activity was measured following the method of Sinha [58]. For this measurement, 50 µg of treated and untreated cell protein (50 µl volume) was added to 100 mM of phosphate buffer (pH = 7.4; 1 ml) containing 200 mM H_2O_2 (0.5 ml) and distilled water (450 µl). The mixture was incubated at 37 °C for 2 min, and the reaction was stopped by adding 5% potassium dichromate/acetic acid solution (in 1:3 ratio). The absorbance was read at 570 nm. The experiments were performed in triplicate.

Protein Estimation

The total protein content was estimated by bicinchoninic acid (BSA) protein assay kit (Lamba Biotech, USA) using bovine serum as a standard.

Morphological Observation

To observe the morphological changes induced by HNE and/or WS, SH-SY5Y cells were pre-exposed to WS at 12.5, 25, and 50 µg/ml for 24 h, and then to 50 µM HNE for 24 h. The changes occurring in the cells were observed under a phase-contrast inverted microscope at 20×.

Caspase-3 Activity

The caspase activity was determined using a commercially available kit (Caspase 3 activity kit, Sigma) as per the instruction provided by manufacturer. In brief, after exposure to HNE and/or WS, the cells were lysed in lysis buffer. The cell extract (50 µg/ml) was incubated for 4 h at 37 °C with DEVD-pNA, a pseudosubstrate used to measure the caspase-3 activity. The free yellow colored pNA released from DEVpNA after cleavage by DEVDase exhibited the quantity of caspase activity. The experiments were performed in triplicate.

ROS Generation

Quantitively and qualitatively ROS production was measured using the 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) fluorescent probe [59]. Dichlorofluorescein was used as the substrate, as it was converted into dichlorofluorescein that was measured. In brief, treated and untreated SH-SY5Y cells were incubated with 20 µM DCF-DA in the dark at 37 °C for 60 min. The relative changes in ROS production were evaluated using a fluorescence microplate reader at 485/530 nm excitation/emission, respectively. The experiments were performed in triplicate. The intracellular fluorescence of DCF was also observed under the fluorescence microscope.

Real-time PCR Analysis of Apoptotic Marker Genes

For analyzing the expression of apoptotic marker genes, 1×10^6 SHSY5Y cells were cultured in six-well plates. Subsequently, the cells were treated with HNE and/or WS for 24 h, after which total RNA was extracted using Trizol reagent (Invitrogen). The integrity and yield of RNA samples were confirmed by gel electrophoresis and spectrophotometry. An equal amount of RNA was used to synthesize cDNA using the reverse transcription kit (Applied Biosystems, USA). Then, RT-PCR^q was performed using the Light-Cycler® 480 instrument. The primer sequences used for the analysis of marker genes have been reported previously [60]. The experiments were performed in triplicate.

Statistical Analysis

All results are presented as the mean ± SD of three separate experiments performed in triplicate. Data were analyzed using one-way ANOVA, and post-hoc Dunnett's test was performed for statistical analyses. Values were considered to be statistically significant at p < 0.05.

Results

WS Protects Against HNE-Induced Cytotoxicity

Figure 1a shows the HNE-induced dose-dependent cytotoxic effects in SH-SY5Y cells. The cell viability was 49% at 50 µM concentration (p < 0.01) of HNE; therefore, this concentration was chosen to induce cytotoxicity in SH-SY5Y cells. As shown in Fig. 1b, that WS at ≤ 100 µg/ml or less could not induce any damage to SH-SH5Y cells treated for 24 h. Hence, we selected the non-cytotoxic concentrations (50, 25, and 12.5 µg/ml) of WS to study the neuroprotective effects against HNE-induced cellular toxicity. To measure the cytoprotective potential of WS against HNE, the three
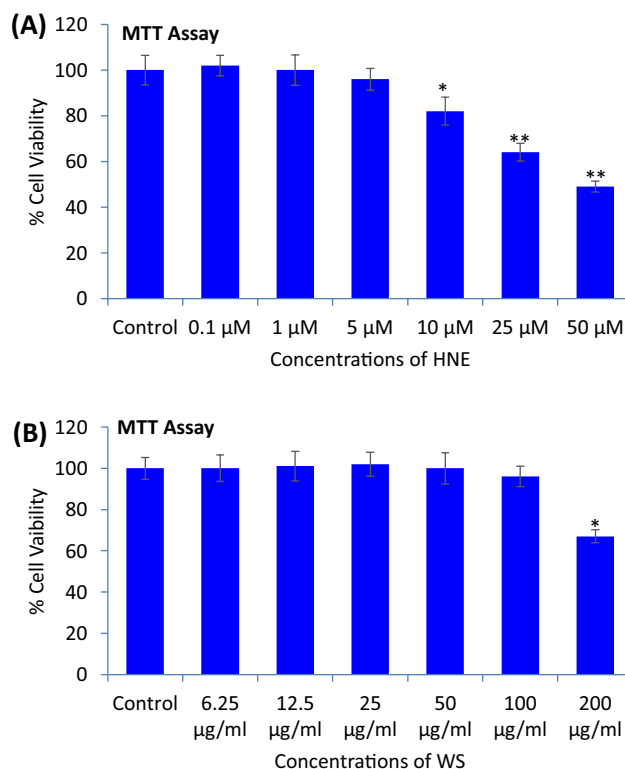

Fig. 1 Concentration-dependent cytotoxicity of **a** 4-Hydroxynonenal (HNE) and **b** *Withania somnifera* (WS) in SH-SY5Y cells exposed for 24 h as measured by MTT assay. Data are presented as the mean ± SD of three separate experiments performed in triplicate. *p < 0.05 and **p < 0.01, compared with control

different endpoints MTT assay, NRU assay and TB assay were employed. SH-SY5Y cells were pre-exposed to WS (12.5–50 µg/ml) for 24 h and then to 50 µM of HNE for 24 h. Results showed that WS increased the viability of SH-SH5Y cells that was reduced by HNE as observed by all three parameters. As shown in Fig. 2, HNE treatment significantly reduced the viability of SHSY-5Y cells; however, pre-exposure to WS significantly increased the HNE-reduced cell viability in a dose-dependent manner. All concentrations (12.5, 25 and 50 µg/ml) of WS were found to increase the viability of SH-SY5Y cells, with a maximum increase of 46% (p < 0.01) at 50 µg/ml as assessed by the MTT assay (Fig. 2a). A lower WS concentration (12.5 µg/ml) showed no significant protection against HNE-induced toxicity, whereas a higher concentration, i.e. 50 µg/ml increased the cell viability by up to 32% as assessed by NRU assay (Fig. 2b). The TB assay also showed a significant increase in the viability of SH-SH5Y cells by up to 39% (p < 0.01) at 50 µg/ml WS as compared to that of cells exposed to HNE only (Fig. 2c). We further examined the effect of HNE exposure on membrane integrity by analyzing the extracellular level of the intracellular enzyme LDH. Leakage of LDH from the cell membrane is a marker of cell death. Our results showed that HNE
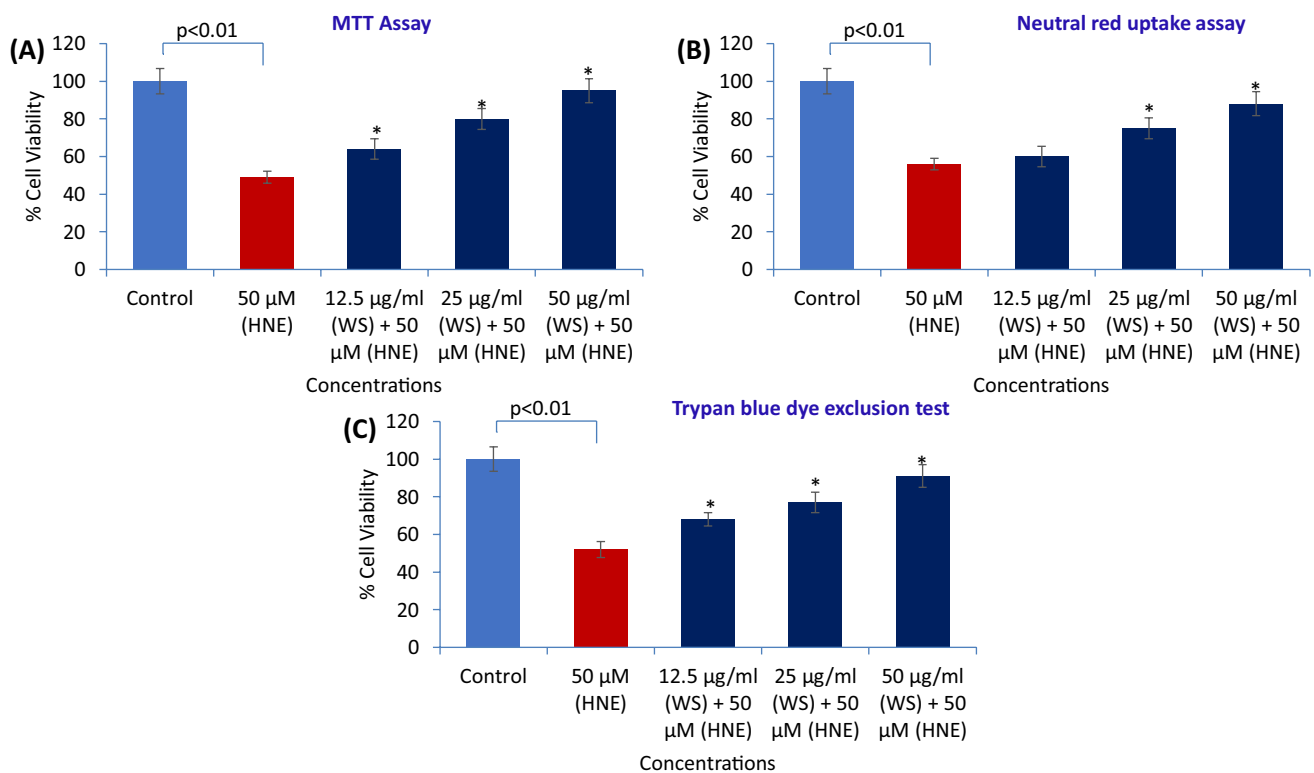
Fig. 2 *Withania somnifera* (WS) attenuated 4-Hydroxynonenal (HNE)-induced cell death in SH-SY5Y cell line. Cells were exposed to 12.5, 25 and 50 μg/ml of WS and then to 50 μM of HNE for 24 h. After incubation, cell viability was determined by MTT assay **a** neutral red uptake assay **b** and trypan blue dye exclusion test **c** Data are presented as the mean ± SD of three separate experiments performed in triplicate. *p < 0.01 vs HNE

significantly increased LDH leakage compared to control (Fig. 3a). However, pretreatment with WS at 12.5–50 μg/ml significantly decreased the level of LDH leakage induced by HNE in a dose-dependent manner (Fig. 3a). In addition, WS completely inversed the inhibition of intracellular LDH level induced by HNE (Fig. 3b).

WS Protects Against HNE-Induced Oxidative Stress

The results of oxidative stress parameters are summarized in Fig. 4. The MDA level was significantly increased by up to 1.8-fold in 50 μM HNE-treated SH-SY5Y cells compared to untreated control (Fig. 4a). The exposure of SH-SY5Y cells to a higher concentration, i.e. 50 μg/ml of WS led to completely reverted the level of MDA induced by HNE-treatment. The GSH level was reduced by 55% after exposure to HNE at 50 μM concentration; however, it was significantly restored by WS treatment in a dose-dependent manner (Fig. 4b). The level of catalase activity in the HNE-treated SH-SY5Y cells was increased by up to 80%, which was significantly decreased by WS treatment in a dose-dependent manner. The reduced catalase values were 12, 36 and 68% (p < 0.01) at 12.5, 25 and 50 μg/ml concentrations of WS, respectively, compared with HNE treatment (Fig. 4c).

WS Prevents HNE-Induced SH-SY5Y Cell Proliferation and Apoptosis

As shown in Fig. 5a, exposure to 50 μM of HNE decreased the proliferation and reduced the progress of SH-SY5Y cells observed under phase-contrast inverted microscope. After 24 h exposure several cells lost their adherence capacity to the surface and detached from the bottom of the plate. However, pre-exposure to WS at 12.5, 25, and 50 μg/ml significantly prevented this loss of SH-SY5Y cells induced by HNE. The caspase-3 enzyme activity in WS- and HNE-treated SH-SY5Y cells was also evaluated by using DEVD peptide nitroanilide pNA (Fig. 5b). Exposure to 50 μM HNE significantly increased the caspase-3 enzyme activity by up to 2.2-fold in SH-SY5Y cells compared to control. Nonetheless, preexposure of SH-SY5Y cells to WS strongly reduced the caspase-3 activity in a dose-dependent manner. Especially, 50 μg/ml WS-treatment was reduced the caspase-3 activity to almost similar to control (Fig. 5b).
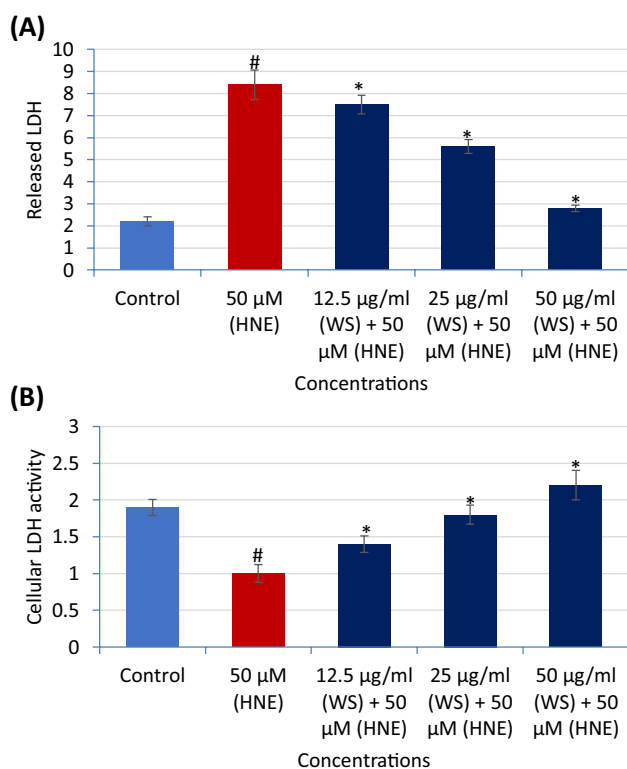
(A)

(B)

Fig. 3 Protective potential of WS against HNE-induced neurotoxicity in SH-SY5Y cells analyzed by **a** LDH leakage assay and **b** Intracellular LDH activity after 24 h exposure of SH-SY5Y cells to different concentrations of WS and then to HNE (50 µM) for 24 h. Data are represented as the mean ± SD of three separate experiments performed in triplicate. #p < 0.01 vs control and *p < 0.01 vs HNE

WS Prevents HNE-Induced ROS Generation in SH-SY5Y Cells

To examine the intracellular ROS production induced by HNE and the preventive potential of WS, quantitative and qualitative analyses were conducted using the 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) fluorescent probe. The qualitative measurement was achieved by grabbing the fluorescence images using a fluorescence microscope, and the quantitative analysis was performed by calculating the cellular fluorescence using a spectrofluorometer. The spectrofluorometric assay showed that HNE-treatment at 50 µM concentration increased the ROS production by up to 220% in SH-SY5Y cells (Fig. 6). However, pre-treatment with WS at 12.5, 25, and 50 µg/ml significantly suppressed the production of intracellular ROS, with a decrease of up to 112% at maximum concentration of 50 µg/ml (Fig. 6b). Furthermore, the effects of HNE and WS were visually confirmed under the fluorescence microscope. SH-SY5Y cells showed an increase in the strength of green fluorescence after HNE treatment at 50 µM. However, the fluorescence intensity was significantly decreased by pre-exposure to WS at 12.5, 25,

and 50 µg/ml, which prevented ROS production (Fig. 6a). These results designated that WS has the potential to scavenge ROS production induced by HNE.

WS Prevents the mRNA Expression of Apoptotic Marker Genes Induced by HNE in SH-SY5Y Cells

Figure 7 shows the expression profile of apoptotic marker genes associated with HNE-induced alterations and the protective potential of WS in SH-SH5Y cells. The mRNA expression levels of apoptotic marker genes p53, caspase-3, and -9, and Bax were upregulated by up to 2.5-, 1.9- and 2.3-, and 1.8-fold, respectively; however, Bcl-2 expression was downregulated by 0.45-fold in SH-SY5Y cells exposed to 50 µM of HNE. Nevertheless, pretreatment of SH-SY5Y cells with 50 µg/ml WS significantly attenuated the increased gene expressions of p53, caspase-3, and -9, and Bax to 1.2-, 1.1-, and 1.2- and onefold, respectively. The downregulated Bcl-2 gene expression was also upregulated by up to 0.95-fold after exposure to WS at 50 µg/ml concentration (Fig. 7).

Discussion

The cytotoxicity and oxidative stress-mediated damages induced by HNE have been reported in various cell types [7–10]. It has also been clearly documented that HNE is associated with various neurodegenerative disorders [61, 62]. The induced level of HNE is known to affect different cellular events such as cell differentiation and proliferation and apoptosis in neuronal cells through tempering the expression of apoptotic marker genes [6]. Our previous research also showed that cytotoxic concentrations of HNE significantly affected the sensitivity of neurotransmitter receptors [17] and induced oxidative stress-mediated apoptotic cell death in PC-12 cells [15, 16]. Therefore, in this study we explored the agent that can protect against neuronal cell injury induced by HNE.

Owing to the increasing interest in naturally derived agents with potential neuroprotective properties to manage neurodegenerative diseases [63], we aimed to identify the neuroprotective effect of WS against HNE-induced neurotoxicity in the SH-SY5Y cell line. WS (Ashwagandha), which has been used since ancient times in the traditional system of remedies, is known for its antioxidant, anticancer, anti-inflammatory, antistress, adaptogenic, antiarthritis and immunomodulatory activities [36–43]. We treated SH-SY5Y cells with various doses of HNE (0.1–50 µM) and WS (6.25–200 µg/ml) for 24 h to ascertain the cytotoxic and biologically safe doses of HNE and WS. We observed that HNE at 50 µM concentration resulted in ~50% cell death and WS at 12.5, 25, and 50 µg/ml concentrations did not produce any
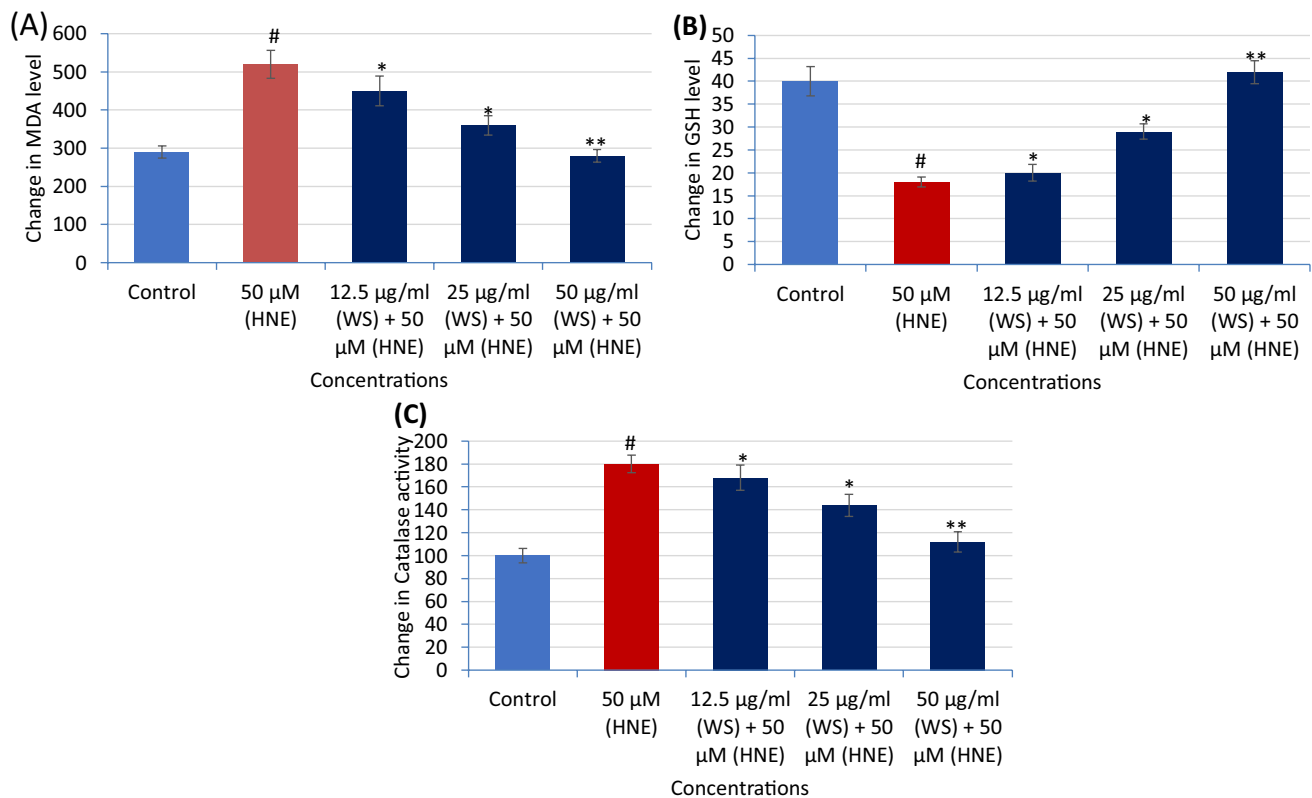
Fig. 4 HNE induced oxidative stress and the protective potential of WS in SH-SY5Y cells. Cells were treated with WS at concentrations of 12.5, 25, and 50 µg/ml for 24 h and then to 50 µM of HNE. At the end of treatment, oxidative stress markers were determined. **a** MDA level, **b** GSH level and **c** Catalase activity. Data are represented as the mean ± SD of three separate experiments performed in triplicate. #$p < 0.01$ vs control and *$p < 0.05$, **$p < 0.01$ vs HNE

damage to SH-SY5Y cells when treated for 24 h. Therefore, we selected the cytotoxic concentration (50 µM) of HNE to induce the cytotoxicity and noncytotoxic concentrations (12.5, 25 and 50 µg/ml) of WS to study the neuroprotective potential against HNE-induced damages.

The MTT, NRU, and TB assays confirmed that HNE at 50 µM concentration induced SH-SY5Y cell death. This could be due to necrosis or apoptotic cell death. Previous studies have also reported HNE-induced cell death in PC12 [17] and SH-SY5Y [6] cell lines at this concentration. Pretreatment with WS at 12.5, 25, and 50 µg/ml concentrations led to a dose-dependent increase in the viability of SH-SY5Y cells. Similarly, Bharathi et al. [64] reported the cytoprotective effects of *Emblica officinalis* against aluminum chloride-induced toxicity in SH-SY5Y cells. Pretreatment with WS extract has been reported to protect against structural changes in spine density induced by morphine in rats [65]. The neuroprotective potential of WS against different toxic insults has also been extensively reported [66], which support our study results. Previous studies suggest that HNE-induced death in SH-SY5Y cells could be due to mitochondrial impairment, and WS is capable of increasing the cell viability by repairing the

mitochondrial activity [3]. These results were consistence with the decline in the extracellular LDH level and the increase in the intracellular LDH level upon pretreatment of SH-SY5Y cells with WS. However, the mechanism(s) through which mitochondrial impairment occurs has not been investigated in detail. There are various other potential routes that could lead to cell death and proliferation.

In this investigation, we examined whether these cytotoxic/cytoprotective responses are due to oxidative stress and variations in the pattern of certain genes responsible for cell death. To understand the protective potential of WS, we examined the various parameters of oxidative damage measurements, i.e. MDA, GSH, and catalase activities. Our results showed that HNE at 50 µM concentration significantly increased the MDA and catalase activity and decreased the level of GSH in SH-SY5Y cells. These results are inconsistent with previous reports on the oxidative stress-inducing capacity of HNE in a variety of cells as well as neuronal cells [6, 15, 67, 68]. However, pre-exposure to WS at 12.5, 25, and 50 µg/ml reverted the levels of MDA, GSH, and catalase in a dose-dependent way. These effects could be due to the presence of
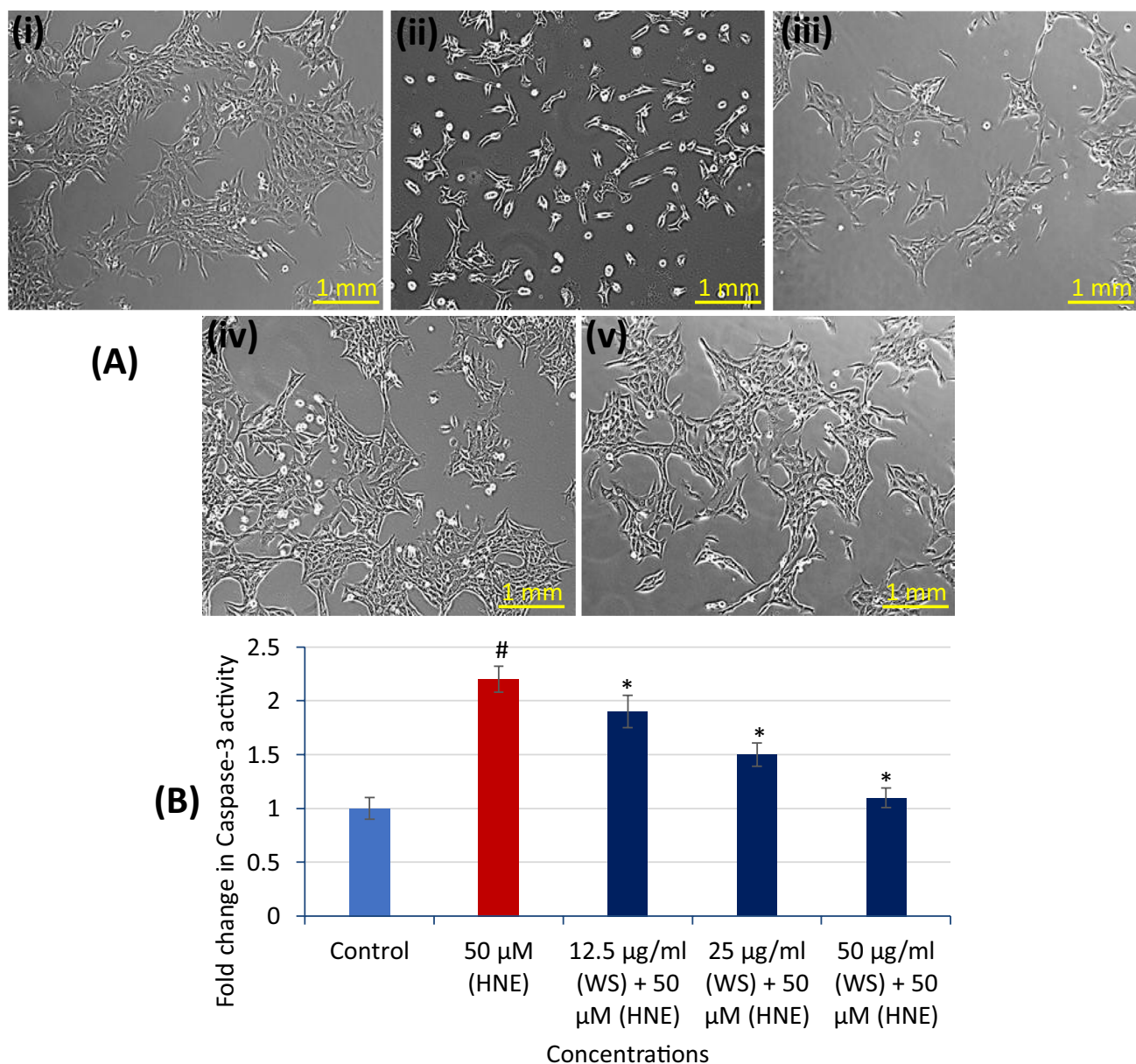
Fig. 5 a Change in the proliferation of SH-SY5Y cells exposed to WS and/or HNE for 24 h. Images were grabbed under inverted light microscope. (i) Control, (ii) HNE (50 μM), (iii) WS (12.5 μg/ml)+HNE (50 μM); (iv) WS (25 μg/ml)+HNE (50 μM), (v) WS (50 μg/ml)+HNE (50 μM). **b** Caspase-3 enzymatic activity in SH-SY5Y cells after the exposure of WS and then to HNE for 24 h. Data are represented as the mean±SD of three separate experiments performed in triplicate. #p<0.01 vs control and *p<0.01 vs HNE. Each scale bar=1 mm

antioxidants in WS. In fact, the antioxidant properties of WS have already been reported [69, 70].

Observation of cell morphology showed that SH-SY5Y cells treated at 50 μM HNE exhibited decreased proliferation and reduced growth, whereas treatment with WS at 12.5, 25, and 50 μg/ml prevented the inhibition and improved the growth and development of SH-SY5Y cells. The enzymatic action of caspase-3 in the cells was evaluated to confirm the HNE-induced apoptosis level. Results showed that HNE at

50 μM concentration increased the caspase-3 activity by up to 2.2-fold in SH-SY5Y cells. Our results are in consistent with other reports showing an increase in caspase-3 activity in PC12 cells when exposed to HNE [16] and other toxicants in SH-SY5Y cells [71]. In the present study, pretreatment with WS at 12.5, 25, and 50 μg/ml decreased the HNE-induced caspase-3 activity, suggesting that WS acts upstream of caspase-3 to block apoptosis. Studies have well documented the HNE-induced cell death through the pathway

Fig. 6 Reactive oxygen species (ROS) generation in SH-SY5Y cell line exposed to different concentrations of WS and then HNE for 24 h. **a** Fluorescence of Intracellular ROS generation grabbed under fluorescence microscope. (i) Control, (ii) HNE (50 μM), (iii) WS (12.5 μg/ml)+HNE (50 μM), (iv) WS (25 μg/ml)+HNE (50 μM); (v) WS (50 μg/ml)+HNE (50 μM). **b** Percent ROS generation in SH-SY5Y cells determined by spectrophotometry. Data are represented as the mean±SD of three separate experiments performed in triplicate. #p<0.01 vs control and *p<0.01 vs HNE. Each scale bar=1 mm

-9, and Bax) and downregulated the expression of antiapoptotic gene Bcl-2. However, pretreatment with WS at 50 μg/ml significantly (p<0.01) downregulated the increased expression level of proapoptotic genes and upregulated that of the antiapoptotic gene. These reverse effects on level of apoptosis-related genes upon WS pretreatment suggested its antioxidative property, which could be arbitrated by the caspase-3 cascade pathway in SH-SY5Y cells.

Conclusion

This study demonstrated that HNE-treatment induced cytotoxicity, oxidative stress and apoptosis in the SH-SY5Y cell line. A dose-dependent cytotoxic effect of HNE was observed in SH-SY5Y cells at 10, 25, and 50 μM concentrations. Pretreatment with noncytotoxic concentrations (12.5, 25, and 50 μg/ml) of WS diminished the cytotoxicity induced by 50 μM HNE in a concentration-dependent way. Treatment with WS at 12.5–50 μg/ml concentrations was also found to diminish the ROS generation and caspase-3
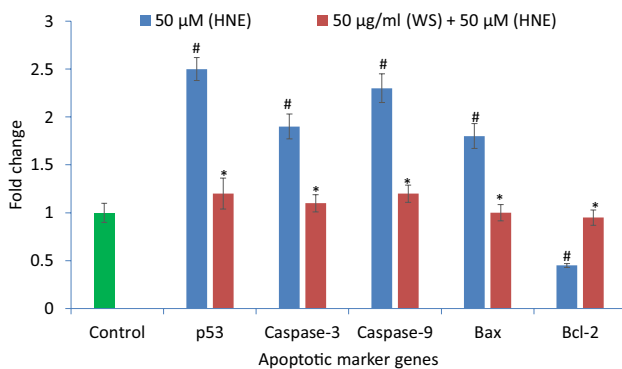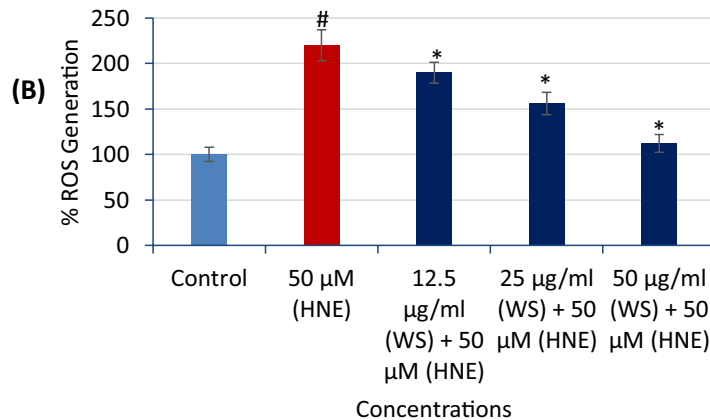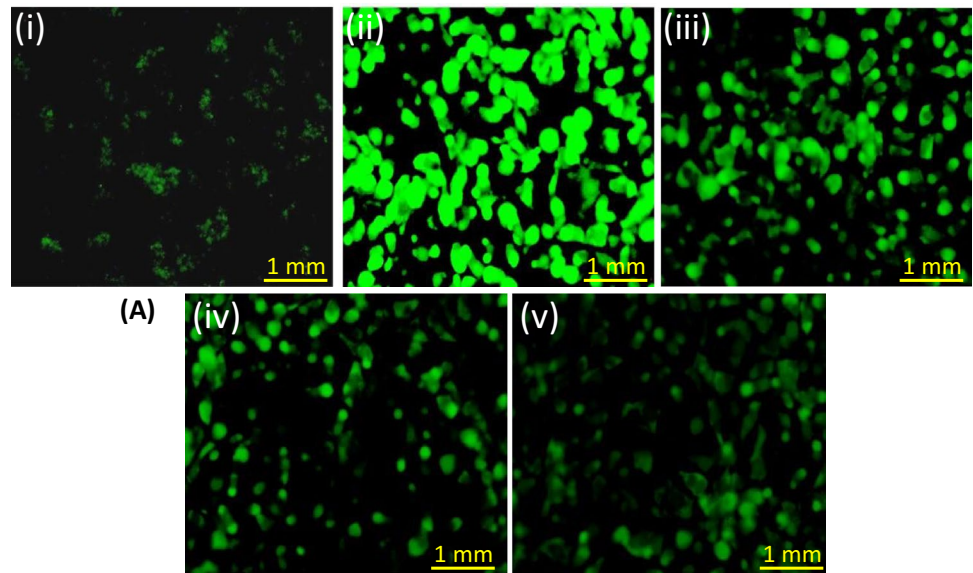

Fig. 7 mRNA expression of p53, Caspase-3, and-9, Bax, and Bcl-2 genes analyzed by real time-PCR in SH-SY5Y cells exposed to WS (50 μg/ml) and/or HNE (50 μM) for 24 h. Data are presented as the mean±SD of three separate experiments performed in triplicate. #p<0.01 vs Control and *p<0.01 versus HNE

of mitochondrial-mediated apoptosis [72, 73]. Our study results revealed that 50 μM HNE upregulated the mRNA expression of proapoptotic maker genes (p53, caspase-3, and

level. The protective effects of WS against HNE-induced oxidative damage and apoptotic cell death provide further evidence regarding the antioxidative and antiapoptotic properties of WS. These properties render this natural agent potentially effective against neurotoxicants such as HNE. The findings of this study could provide novel understanding for the development of beneficial agents in the management of neurodegenerative disorders.

Author Contributions MAS and ABP conceived and designed the research. MAS, NNF and MMA conducted the experimental work. ABP and AAA provided reagents and tools in the laboratory. MAS, ABP and AAA analyzed the data. MAS and NNF wrote the manuscript. All authors have read and approved the manuscript.

Compliances with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical Approval This paper does not contain any studies with human or animals.

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